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(54) Title: ISOLATION OF GLUCAN PARTICLES AND USES THEREOF

(57) Abstract: The present invention relates to the isolation of novel glucan particles but also to mannoprotein from natural sources such as yeast cell walls, novel isolation methods, and the use of products thereof.

## Isolation of Glucan Particles and uses thereof

### Field of Invention

5 The present invention relates to the isolation of novel glucan particles from natural sources such as yeast cell walls, novel isolation methods, and the use of products.

### Background

10 The cell walls of unicellular organisms and of plants mainly consist of polysaccharides, partly associated with proteins. Important functions are: Rigidity, physical protection of the cells, osmotic stability, selective permeability support, cell/cell adhesion, binding of compounds and extracellular enzyme support. Since the polysaccharides form a three dimensional network, cell walls may serve as a resource for particles that maintain certain useful properties of the native cell wall such as selective adsorption capacity. The final properties of the particles depend on the starting material (e.g. yeast strain or other microbial or plant cells) and on the level of preservation of the structural integrity during isolation.

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20 Therefore, a prerequisite for the isolation of functional particles are non-denaturing isolation procedures.

25 In the following the structure and composition of the yeast cell wall, an important potential resource of said particles, is outlined.

30 The precise structure and composition of the yeast cell wall strongly depends on the type of yeast strain and culture conditions (R. Bonaly, H. Moulki, A. Touimi Benjellouen, M. Pierrefitte, Biochim. Biophys. Acta 244, 484 (1971)). A shortage of amino acids, for example, reduces the protein content in the cell wall. Yeasts are unicellular organisms with a rigid cell wall made of polysaccharides. The cell shape is oval to round with an average diameter of 5 - 13  $\mu\text{m}$ . The cell walls show a thickness of about 70 nm and account for 15 - 25 % of the yeast dry weight (J.S.D. Bacon, V.C. Farmer, D. Jones, I.F. Taylor, Biochem. J. 114, 557 (1969)). As mentioned, the overall composition of the cell wall varies

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and depends on the special strain and on culture conditions. This forms the basis for the isolation of a great number of cell wall particles with different properties.

5 In general, the main components of the yeast cell wall are mannan (typically about 30 % by weight), glucan (also about 30 % by weight), protein (15 % by weight), lipids (about 10 % by weight) and chitin (about 2 % by dry weight). The latter is exclusively located in the budding scar of the yeasts.

10 *The mannoprotein component*

By definition mannan, is a polymer that is exclusively composed of mannose units. In yeasts, mannan is associated with protein in both, the external surface of the yeast cell wall, as a mucigenous polysaccharide 15 and in the inner cell membrane. It accounts for 20 - 50 % of the dry weight (C.E. Ballou, *Adv. Microbiol. Physiol.* **14**, 93 (1976)). Mannan is linked to a core-peptide chain as an oligomer or polymer (R. Sentandreu, D.H. Northcote, *Biochem. J.* **109**, 419 (1968)). The complex consists of 5 - 50 % proteins. Oligomeric mannan is bonded directly to serine and threonine (R. Sentandreu; D.H. Northcote, *Carbohydr. Res.* **10**, 584 (1969)) whereas polymeric mannan is bonded to asparagine via 20 N-acetylglucosamine. The many individual aspects relating to the mannoprotein complex, including that the mannose units are linked by  $\alpha$ -1,6,  $\alpha$ -1,2 and  $\alpha$ -1,3-linkages were compiled and reviewed by Ballou et al. 25 (C.E. Ballou, *Adv. Microbiol. Physiol.* **14**, 93 (1976); C.E. Ballou, *Adv. Enzymol.* **40**, 239 (1974)).

*The glucan component*

30 Glucan is a glucose polymer and accounts for 30 - 60 % of the dry weight. The majority of the polyglucoside is linked via  $\beta$ -1,3 glycosidic linkages and only 10 - 20 % by  $\beta$ -1,6 glycosidic linkages (S. Peat, J.R. Turvey, J.M. Evans, *J. Chem. Soc.* 3868 (1958)). If glucan is treated 35 with approximately 3 % caustic soda at 75 °C, a maximum of one-third of the glucan is solubilized (J. S. Bacon, V. C. Farmer, D. Jones, *Biochem. J.* **114**, 557 (1969)). Consequently the glucan is divided into (1) an alkali insoluble fraction (glucan A), and (2) an alkali soluble fraction

(glucan B) (G.H. Fleet, D.J. Manners, *J. Gen. Microbiol.* **94**, 180 (1976)).

5 Glucan A accounts for 80 - 85 % of the cell wall glucan and consists primarily of  $\beta$ -1,3 glycosidic linkages as well as of about 3 %  $\beta$ -1,6 glycosidic linkages. 80 - 85 % of the glycosidic linkages of glucan B (15 - 20 % of the total glucan) are  $\beta$ -1,3 and 8 - 12 % are  $\beta$ -1,6 glycosidic linkages. 3 - 4 % of the glucose units are branchings. The  $\beta$ -1,6 glycosidic linkages are selectively hydrolysed by acetylosis. It is proposed that the  $\beta$ -1,3 glucan chains are linked via  $\beta$ -1,6 intermediate chains (J.S.D. Bacon, V.C. Farmer, D. Jones, *Biochem. J.* **114**, 557 (1969)).  
10 Using electron microscopy it was possible to demonstrate a fibrillar structure for the  $\beta$ -1,3 component and an amorphous structure of the 1,6 component (M. Kopecká, *J. Basic Microbiol.* **25**, 161 (1985)).

15 *Chitin and lipid components*

20 Chitin (N-acylated poly-glucosamine) is located exclusively in the budding scars, where it forms a ring (E. Cabib, B. Browers, *J. Biol. Chem.* **246**, 152 (1971)). As a lipid compound dolichol phosphate was isolated from the cell walls (P. Jung, W. Tanner, *Eur. J. Biochem.* **37**, 1 (1973)).  
The rest of the lipid component consists of glycerol esters of various fatty acids.

25 *The Structure of the yeast cell wall*

30 Electron microscopic investigation of the process of biosynthesis and assembly of the glucans in *Candida albicans* reveals the development of the fibrous network of the cell wall. The triple helices which appear as microfibrils with a diameter of approx. 2 nm are self-assembled end-to-end and side by side and are twisted together leading to fibrils of 4 - 8 nm in diameter. These fibrils finally associate to flat ribbon-shaped bundles, 8 - 16 nm thick and 100 - 200 nm wide and thus form the basic network structure of the cell wall. The interfibrillar spaces of the network at this stage have dimensions of about 100 - 200 nm and most likely mark the origin of the pores which are present in the cell wall at the final stage and which constitute the structural basis for their ability to adsorb compounds with great significance in a large number of different areas.

They are gradually filled with the additional components and manno-proteins which are known to form anchors to the membrane lipids.

*Isolation of yeast cell wall components*

5 Fractionation of the cell walls, as e.g. of *Saccharomyces cerevisiae* starts either from whole cells or from cell walls e. g. obtained by autolysis; both starting materials may be used in dry or wet form. In some cases the cells or cell walls are pre-treated mechanically (by sonification or by treatment with glass beads). The starting material as well as the mechanical disruption greatly influence the purity of the resulting fraction. A large number of different methods were reported for the isolation of cell wall components (F.M. Klis, Yeast 10, 851 (1994)). They can be grouped (1) in methods for the isolation of mannoprotein, and (2) in methods for the isolation of glucan.

10 15 A common reagent of chemical methods for the isolation of mannoprotein is sodium hydroxide of varying concentrations and using a wide range of temperatures and treatment times (Int. Patent WO 94/04163 (1994); D.L. Williams, R.B. McNamee, E.L. Jones, H.A. Pretus, H.E. Ensley, I. Williams, N.R. Di Luzio, Carbohydr. Res. 219, 203 (1991)).

20 Depending on the reaction conditions, such treatments also solubilize more or less glucan (see above definition of soluble and insoluble glucan). In some cases, organic bases like ethylene diamine and buffers like citrate salts find application to solubilize mannoproteins (R. Sentandreu, D.H. Northcote, Biochem. J. 109, 419 (1968); T. Nakajima, C. Ballou, J. Biol. Chem. 249, 7679 (1974)). Extraction with a 2 % boiling sodium-dodecyl-sulfate (SDS) in the presence or absence of reducing agents, like mercaptoethanol, represents a widely used approach to free glucan from mannoproteins and other proteins (E. Valentin, E. Herrero, F.I.J. Pastor, R. Sentandreu, J. General Microbiol. 130, 1419 (1984); F.I.J. Pastor, E. Valentin, E. Herrero, R. Sentandreu, Biophys. Acta 802, 292 (1984)). Treatment of whole cells with pure water at temperatures of up to 135 °C was also applied, yielding a highly contaminated mannoprotein fraction (S. Peat, W. J. Whelan, T.E. Edwards, J. Chem. Soc. 29 (1961); N. Shibata, K. Mizugami, S. Susuki, Microbiol. Immunol. 28, 1283 (1984); Y. Okubo, T. Ichikawa, S. Susuki, J. Bact. 136, 63 (1978)).

25 30 35

Enzymatic methods were alternatively used for releasing the manno-proteins. For this purpose, proteases and glucanases are used, acting on the protein part of the mannan or the glucan fixing the mannoprotein ( $\beta$ -1,6 glucan).

5

The mannan-free glucan is further purified by procedures that include acid treatment such as acetic acid or HCl.

10

The summarised chemical procedures for isolation and purification of cell wall components will more or less affect the nativity of the polymers, which is primarily reflected in the occurrence of increased amounts of soluble glucan and in a disturbance of the structure of the insoluble glucan fraction. It is especially the latter negative impact of existing glucan isolation procedures that make the insoluble glucan less suitable for adsorbent applications. When such chemical treatments are used under milder conditions, the pores of the glucan skeleton are not properly activated, i.e. freed from physically or chemically bound pore filling material. This also yields insoluble glucan not optimal for adsorption.

15

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Therefore, it is the objective of this invention to provide simple and effective methods for the isolation of glucan particles, which are characterised by a native structure and active pores.

25

The respective process for isolating glucan particles with such features from cells, cell walls, or cell wall fragments of unicellular organisms like yeasts or fungi or of cell wall residues of glucan-containing plants, comprises the steps a) – c) which may be proceeded in any sequence (Fig. 1). These steps are characterised as follows:

30

a) extracting mannoproteins with water at temperatures above the boiling point of water from suspensions,

b) removal of contaminating proteins with protease or non-denaturing chemical means,

35

c) removal of contaminating lipids with lipases or by solvent extraction.

The extraction of step is preferably proceeded with adjusted pH under elevated pressure.

Depending on the starting material one or more steps may be deleted.  
5 Cells, cell walls or cell wall fragments of e. g. yeast or fungi or of unicellular organisms other than yeast or cell wall residues of glucan-containing plant tissues are used as starting material.

According to the invention these new glucan particles are obtainable  
10 from these starting materials by combining steps c) and b)  
or  
steps b) and c) respectively.

As far as appropriate, residual non-glucan components may be removed by non-denaturing chemical means, such as extraction of  
15 non-glucan residues with NaOH at low concentration and temperature, with 2 % sodium dodecylsulfate solution at elevated temperature, and with organic solvents, such as acetone, at room temperature or at elevated temperatures.

20 If useful starting materials are treated according to the invention and if the steps of the inventive process are carried out in a suitable sequence the basic structure of the isolated glucan particles remains intact and shows properly active pores. Naturally this means that each single step of the process has to be adapted to the treated material.

25 It has been found that glucan particles can be isolated with preserved porous and fibrous structural properties. These particles are having a molecular weight ( $M_w$  or  $M_n$ ) of more than 100 000, especially more than 400 000, having essentially retained its native structure after solubilization by carboxymethylation. Their particle sizes are in a range of  
30 0.1 to 25 micrometers, preferably 0.5 to 15 micrometers, and most preferably 2 to 10 micrometers. In contrast to known glucan these glucan particles are insoluble in water and in most of the known organic solvents, while they are having activated pores and are showing an increased stability at high pH-values. Additionally, these glucan particles  
35 are able to form stable gels; for example they are forming a stable gel

in organic solvents or acidified water, when an aqueous suspension of these particles is heated to a temperature higher than 80 °C.

Glucan particles according to the present invention may be obtained by extracting mannoproteins from an aqueous cell wall suspension in the concentration range of 1 – 20 % by weight, preferably 10 – 20 %, most preferably 13 – 17 % with adjusted pH in the range of 5 – 9, preferably pH 6 – 8, most preferably pH 6.5 to 7.5, at a temperature in the range of 100 – 150 °C, preferably 110 – 140 °C, most preferably 120 – 130 °C, for a defined period of time, e. g. 3 – 7 hours, under elevated pressure.

According to the described treatment lipid and protein containing glucan particles are obtained as solid fraction and mannoprotein as the soluble fraction, which may be isolated.

The extracted mannoprotein can be isolated from the aqueous solution by precipitation. It is found, that this precipitation can be induced by mixing the solution with an alcohol. Suitable alcohols are short chained alcohols. Preferably an alcohol selected from the group methanol, ethanol, propanol or butanol is used. Most preferably the precipitation is carried out with ethanol. Alternatively the mannoprotein may be concentrated by ultra-filtration before precipitation with alcohols.

Lipid and protein containing glucan particles, removed by centrifugation or filtration, are subsequently treated with a protease at pH values and temperatures required for optimum protease activity for 1 – 12 hours, preferably 3 – 8 hours and most preferably 4 – 6 hours. Lipid containing glucan particles result from this treatment and can be isolated.

For obtaining intact mannoprotein the protease treatment on the glucan particles has to be done after the mannoprotein fraction has been separated.

Lipid containing glucan particles removed by centrifugation or filtration, are subsequently treated with a lipase at pH values and temperatures required for optimum protease activity for 1 – 12 hours, preferably 1 – 5

hours and most preferably 2 – 4 hours. From the treatment glucan particles result and may be isolated.

5 Alternatively lipid containing glucan particles may be obtained from lipid and protein containing glucan particles by extraction of protein containing contaminants either with aqueous alkaline solutions such as earth alkali hydroxide, like NaOH, sodium carbonate and sodium hydrogen carbonate solutions at low concentration and low temperature or with sodium dodecylsulfate at a concentration in the range of 0.1 – 5 % by weight, preferably 1 – 3 % by weight, most preferably 1.5 to 2.5 % by weight.

10 Glucan particles may alternatively be obtained from lipid containing glucan particles by cold or hot organic solvent extraction with solvents that are miscible with water, e. g. selected from the group acetone, ethanol, methanol, isopropanol and butanol or mixtures thereof,

15 or with solvents that are not miscible with water, e. g. selected from the group dialkylketones, e.g. isobutylmethylketone, hydrocarbons, e.g., hexane, chlorinated hydrocarbons, e.g. chloroform, methylenchloride, tetrachloroethylene and ester solvents, e.g. ethylacetate, or mixtures thereof,

20 or with mixtures of organic solvents that are miscible with water with organic solvents that are not miscible with water, e. g. methanol/chloroform in a ration of volume of 1 : 1 or hexane/isopropanol in a ration of volume of 3 : 2;

25 or with supercritical fluids, e.g. supercritical CO<sub>2</sub>;

or with supercritical CO<sub>2</sub> and organic solvents as modifiers.

30 Therefore, the process for isolating insoluble native glucan particles with properly activated pores from cells, cell walls, or cell wall fragments of unicellular organisms like yeast or fungi or of cell wall residues of glucan-containing plants, comprises the steps a) – c) mentioned above which may be proceeded in any sequence. These steps are characterised as follows:

35 a) extracting mannoproteins with water at temperatures above the boiling point of water from suspensions with adjusted pH under

elevated pressure,

b) treatment with proteases after pH adjustment at high level and removal of proteins,

c) treatment with lipases after pH adjustment at high level and removal of contaminating lipids,

5 or if appropriate by solvent extraction.

10 Optionally non-glucan residues can be removed by non-denaturing chemical means and as already said above depending on the starting material one or more steps may be deleted.

15 10 Thus glucan particles are prepared which are insoluble in water and most of the common solvents, especially most of the common organic solvents. A unique advantage is their stable three-dimensional structure, which is nearly unchanged in the presence of adsorbed substances or if their surface reacts with active groups. The particles according to the invention possess activated open pores.

20 20 A valuable by-product of the present process is a mannoprotein, which may be recovered from step a) of the process. For this purpose the recovered aqueous fraction of step a) is added to and mixed with an alcohol. This alcohol may be a short-chained alcohol, especially one of the group methanol, ethanol, propanol and butanol. The extracted mannoprotein may be recovered after precipitation by cooling for several hours.

25 25 Distinguishing properties of glucan particles isolated according to the present invention are also:

1. Solubility in DMSO

30 30 Glucan particles according to the invention swell markedly in DMSO but can easily be centrifuged which means that they are not truly dissolved. For comparisons, glucan particles isolated by harsh conditions (conventional glucans) dissolve in DMSO (e.g. D.L. Williams, H.A. Pretus, H.E. Ensley, I.W. Browder, Carbohydr. Res. 253, 293 (1994)) which allows the characterisation in solution like the chromatographic determination 35 of the molecular weight.

## 2. Swelling / Gel formation

5 After heating to a temperature higher than 80°C and subsequent cooling of an aqueous suspension, glucan particles prepared as described above swell and yield a voluminous gel. This gel is stable for several years when it is stored in organic solvents like methanol or acidified water.

## 3. Stability at high pH-values

10 Glucan particles as described are much more stable at high pH values (>pH 10) as compared to conventional glucans, which are solubilized at high pH values.

## 4. Molecular weight

15 Glucan particles as described are insoluble in water and in common organic solvents. Therefore, for the determination of the molecular weight it is necessary to solubilize the glucan particles by an as much as possible mild derivatization method. For example, carboxymethylation under common, only slightly degrading conditions (alkaline isopropanol, chloroacetic acid) yields a water soluble product. Analysis of the product by FFFF (flow field-flow-fractionation) resulted in  $M_w = 880000$  and  $M_n = 581000$ . (Comparison:  $M_w = 35300$  and  $M_n = 35000$  for underderivatized glucan (D.L. Williams, H.A. Pretus, H.E. Ensley, I.W. Browder, Carbohydr. Res. **253**, 293 (1994));  $M_w = 110000$  and  $M_n = 25000$  for glucan 20 phosphate (D.L. Williams, R.B. McNamee, E.L. Jones, H.A. Pretus, H.E. Ensley, I.W. Browder, N.R. Di Luzio, Carbohydr. Res. **219**, 203 (1991)).

## 5. Microscopy

30 Microscopy techniques were used to show the structural features of the glucan isolated according to the invention. Electronmicrography shows the porous surface (Fig. 2) and confocal fluorescence microscopy demonstrates the shape and size of the glucan particles (Fig. 3 and 4).

## 6. Particle size distribution

By means of light scattering a particle size distribution has been determined (Fig. 5).

5 Determined particle sizes of the prepared new glucan are in a size range of 0.1 to 25 micrometers. Most of the particles show particle sizes in the range of 0.5 to 15 micrometers, especially in the range of 2 to 10 micrometers.

10 7. Determination of purity

For the determination of the purity of distinct fractions of glucan particles, the elemental composition of the main possible components are used:

15 Pure glucan: C 44.45 H 6.22  
Pure mannan: C 44.45 H 6.22  
Triglycerides: C ~72 H ~14  
Protein: C ~53 H ~6.5 N ~17

20 In summary the preparation of glucan particles is characterised by the following steps:

25 a) Preparation of protein- and lipid-containing glucan particles from glucan containing starting material, in particular from yeast cell walls by heating an aqueous suspension of yeast cell walls for several hours at elevated temperature above the boiling point of water under elevated pressure.

b) Preparation of lipid-containing glucan particles by treating particles from step a with proteases

30 c) Preparation of glucan particles by treating lipid-containing glucan particles from step b) with lipases

or

35 preparation of glucan particles by combining steps b and c or steps c and b respectively.

or

in variation of the lipase treatment: solvent extraction, e.g. with acetone.

5

Therefore, the described products are useful in a wide range of applications: as carrier in cosmetic or pharmaceutical formulations, as additive for feed and food, as adsorbent for toxic environmental compounds, as active material in chromatography or for immobilisation of substances in different fields of application, such as biotechnology as well as in chemical processing. Glucan particles according to the invention may be used for the formulation of products needed in agriculture in particular in crop protection, since these products have a potential as health promoting agent for animals and humans. Glucan particles according to the invention are also useful for the preparation of pharmaceutical formulations with immune system activating properties as well as for formulations with anti tumour activity or for administration in combination with chemotherapy or dialysis. Since materials prepared according to the present invention are able to stimulate the activity of the immune system, these glucan particles can be used to prepare pharmaceutical formulations to improve the host-defences to bacterial or virus infections as well as such with glucose regulating effect or with improving influence on cardiovascular diseases, in treatment of HIV, and other auto immune conditions like arthritis, lupus, allergic asthma, multiple sclerosis and so on. They are also useful for the preparation of pharmaceutical formulations with prophylactic activity against diseases of age and such with cholesterol reduction activity.

30 As health promoting agent for animals and humans glucan particles of the present invention may be contained in food supplement or dietary compositions.

35 They can be taken or administered to warm blooded mammals in need thereof in various forms such as dried powder mixed with liquid, as a pill, tablet or capsule as part of other formulations for a regulated diet. In addition to the inventive compounds, a variety of fillers, flavouring

agents, binders, minerals and vitamins as well as typical adjuvants used in the art can be used for the preparation of the administration forms. Sorbitol as a sweetener can be mentioned as well as dicalcium phosphate and magnesium stearate as mineral agents are also suitable.

5

Glucan particles according to the invention, that can be isolated as a powder, may be used as food or dietary supplement, which can be used in conjunction with a dietary plan.

10

In preparing the dietary products of the invention, a dry granulation technique may be used that is well understood in the art. Typical equipment used is a roll compactor known as a "Chilsonator" made by the Fitzpatrick Company. The Chilsonator densifies the blended powder by passing the powder between high pressure rollers, which compresses the powder and removes the air. The densified material is reduced to a uniform granule size and can be compressed into tablets after addition of a lubricant in accordance with conventional practice. The blending of the dehydrated powdered glucans and other ingredients and conventional excipients can be carried out with a powder blending machine. This equipment is well known in the art.

20

The food supplement, dietary and pharmaceutical compositions of this invention will contain glucan particles, which can be isolated according to the described process, together with a solid or liquid pharmaceutically acceptable non-toxic carrier. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatine, malt, rice, flour, chalk, silica gel, magnesium carbonate, magnesium stearate, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like. These compositions can take the form of tablets, pills, capsules, powders, sustained-release formulations and the like. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain an effective therapeutic amount of the active ingredients together with a suitable amount of carrier so as to provide the form

for proper administration to the host. These formulations may also contain mannoprotein as such or in combination with glucan particles according to this invention.

5 The compositions of this invention can further include a filler, flavouring agent, binder, mineral, vitamin as mixtures thereof. Tablets can be coated with a film and/or colour layer for protection and colour as is known in the art. These additives are conventional components of dietary products.

10 It has also been found, that the isolated mannoprotein from the hot water treatment can be used in the same manner as the inventive glucan particles for food or pharmaceutical applications. Most advantageously such formulations are prepared using the inventive glucan particles in combination with this mannoprotein.

15 Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilise the present invention to its fullest extent. The preferred specific embodiments and examples of the following are, therefore, to be construed as merely illustrative, and not limitative of the disclosure in any way whatsoever.

20 The entire disclosures of all applications, patents, and publications cited above and below are hereby incorporated by reference.

#### **Example 1:**

##### **25 Preparation of glucan particles**

###### **a) Hot water treatment**

30 Dry yeast cell walls (1.3 kg) were suspended in water (8.7 l) and the suspension was adjusted to pH 7 by adding an aqueous sodium hydroxide solution (30 % w/w). The suspension was heated to 120 °C and stirred for 5 h. After cooling, the suspension was diluted with water (7.3 l) and centrifuged (20 min, 4000 rpm). The supernatant was removed and the sediment resuspended in water (total weight: 17 kg). After centrifugation and removal of the supernatant, the sediment was ready for 35 the next step.

For analytical purposes, an aliquot of the sediment was several times washed with water and centrifuged until the supernatant was colourless and clear and then freeze-dried yielding a pale yellow powder.

5 Yield: 63.6 %  
Protein content: 7.9 %  
Lipid content: 16.3 %

Elemental analysis: C 50.5 %, H 7.5 %, N 3.3 %

10 **b) Protease treatment**

The washed sediment from step a) was resuspended in water to a final volume of 10 l. Then the suspension was heated to 45 °C and adjusted to pH 10.5 by adding an aqueous sodium hydroxide solution (30 % w/w). Savinase (7.5 ml) was added at  $t = 0$ , 1.5 and 3 h. After an overall duration of 5 h, the suspension was neutralised with acetic acid (100 %) and centrifuged (30 min, 4000 rpm). After removal of the supernatant, the sediment was ready for the next step.

20 For analytical purposes, an aliquot of the sediment was several times washed with water and centrifuged until the supernatant was colourless and clear and then freeze-dried yielding a pale yellow powder.

25 Yield (referring to starting material): 31.6 %  
Protein content: 3.5 %  
Lipid content: 17.3 %  
Elemental analysis: C 49.7 %, H 7.6 %, N 1.2 %

**c) Solvent treatment**

30 The moist sediment from step b) was treated with a large excess of acetone and filtered. The residue was washed several times with acetone until the filtrate was colourless and clear and then dried yielding a pale yellow powder.

35 Yield (referring to starting material): 25.7 %  
Protein content: 4.2 %  
Elemental analysis: C 46.2 %, H 6.7 %, N 1.6 %

### Preparation of mannoprotein

5 The first supernatant from step a) was added to ethanol (95 %) under stirring until the water content reached 30 %. The mixture was stored over night at 5 °C leading to a precipitate. The precipitate was filtered, washed several times with ethanol and then dried yielding a white powder.

Yield (referring to starting material): 14.0 %

10 Protein content: 15.4%

Elemental analysis: C 43.1 %, H 6.2 %, N 3.7 %

<sup>1</sup>H and <sup>13</sup>C NMR spectra are shown in Fig. 6 and 7.

### Example 2:

#### 15 Preparation of glucan particles on pilot scale

##### a) Hot water treatment

20 150 kg yeast cell walls, washed free of water-soluble components, were suspended in 850 l of tap water, and the pH was adjusted to 7. The suspension was heated to 125 °C under stirring adapting the stirrer speed accordingly in order to prevent heat gradients and especially local overheating, which leads to gelation. The overpressure amounted to approximately 1.3 bar. After 5 h the suspension was cooled to 45 °C.

25 Vacuum formation was prevented during cooling by opening an air inlet valve equipped with a sterile filter. The lipid and protein containing glucan particles were separated by centrifugation (Westfalia SB 07 centrifuge) and washed twice with water. The washed sediment was used in the next step.

##### 30 b) Protease treatment

35 Lipid and protein containing glucan particles were resuspended in a total volume of 470 l of tap water with a temperature of 45°C. The pH was adjusted to 10.5 with a 30 %-NaOH solution. Then 3.5 l of proteolytic enzyme solution SAVINASE 16.0 L EX (Novo) containing 0.4 l of SAVINASE adapted detergent solution (according to manufacturer

specifications) were added with stirring. After 3 h the pH dropped to 9.5 indicating protein hydrolysis. Therefore, the pH was readjusted step-wise to 10.5 and incubation was carried on until pH remained constant (~2 h). After neutralisation lipid containing (protein free) glucan particles were harvested by centrifugation and washed twice with water. The washed sediment was used in the next step.

For analytical purposes, a small amount of the sediment was treated with an excess of acetone and filtered. The residue was washed three times with acetone and subsequently dried.

Elemental analysis: C 45.1 %, H 6.3 %, N 1.2 %

$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra including the assignment of the signals are shown in Fig. 8 and 9.

15 **c) Lipase treatment**

10 l of the 470 l hot water extracted, protease treated cell wall suspension were further treated with 100 g of LIPOLASE 100 L EX (Novo) containing 4 ml of LIPOLASE adapted detergent solution (according to manufacturer specifications) at 45 °C and pH 10.5 with stirring for three 20 hours. The (protein and lipid free) glucan particles were harvested by centrifugation, washed twice and lyophilised.

25 **Example 3:**  
**Variation of protease treatment**

Yeast cell walls were suspended in water to a final concentration of 11 %. The pH was adjusted to 10.5 by adding an aqueous sodium hydroxide solution (30 % w/w). The suspension was heated to 50 °C under stirring and the reaction was started by the addition of Savinase (3 ml/l suspension). As a standard procedure the addition of equal amounts of Savinase was repeated after 1 and 3 h, respectively. Total incubation time was 4 h. The pH was kept at 10.5 throughout the incubation by addition of adequate amounts of sodium hydroxide whenever necessary. 30 Finally the suspension was neutralised by adding acetic acid and the product was isolated by centrifugation (20 min, 5000 rpm). The sedi- 35

ment was washed twice with water and fats were removed by acetone treatment of the moist sediment as described in Example 1. After drying the protein content was analysed and the results are summarised in table 1.

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**Table 1: Lipid extraction of dry cell walls.**

	Variation of protease treatment	Protein content [%]
5	Standard procedure	4.5
	Additional washing after every protease step	
10	Overnight incubation after third addition of Savinase	2.4
	Additional treatment with 2 % SDS overnight after third Savinase step	1.2
15		

**Example 4:****20 Lipid extraction of dry cell walls with organic solvents**

10 g of dry cell walls (named A containing 16.1 % of lipids and B containing 13.4 % of lipids) were suspended in 200 ml of an organic solvent. The suspension was heated at reflux for 2 h. After cooling to 40 °C, the mixture was filtered. The residue was washed two times with 30 ml of warmed solvent and subsequently dried yielding a colourless or slightly yellowish powder. The combined filtrates were evaporated yielding brownish oil. Table 2 summarises the results of the extractions.

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Table 2: Lipid extraction of dry cell walls.

5	Solvent	cell	lipid con-	extracted
		walls	tent (%)	lipids content (%)
10	n-Hexane/methanol 4:1 (v/v)	A	16.1	15.5 96 %
15	Ethanol (techn.)	"	"	14.3 89 %
	Methanol (abs.)	"	"	14.7 91 %
	Ethanol (techn.)	B	13.4	10.9 81 %
	Methanol (abs.)	"	"	10.9 81 %

**Example 5:****Lipid extraction of spray-dried lipid containing glucan particles with organic solvents**

10 g of spray-dried lipid containing glucan particles (from Example 1: lipid content: 28 %) were suspended in 200 ml of an organic solvent. The suspension was heated at reflux for 2 h. After cooling to 40 °C, the mixture was filtered. The residue was washed two times with 30 ml of warmed solvent and subsequently dried yielding a colourless or slightly yellowish powder. The combined filtrates were evaporated yielding dark brown oil. Table 3 summarises the results of the extractions.

Table 3: Lipid extraction of spray-dried lipid containing glucan particles.

5	Solvent	extracted lipids	
		content (%)	total (%)
n-Hexane/methanol 4:1 (v/v)	28	100	
n-Hexane	2	7	
n-Hexane/isopropanol 4:1 (v/v)	5	17	
10 Isopropanol	12	43	
Acetone (tech.)	10	37	
Acetone/H <sub>2</sub> O 4:1 (v/v)	27	96	
Ethanol (abs.)	27	96	
15 Ethanol (tech.)	27.5	98	
Methanol (abs.)	28	100	

20 **Example 6:**

**Adsorption by glucan particles**

30 - 70 mg of glucan particles were homogeneously suspended in 100 ml of distilled water. The compound of interest was added as stock solution to the glucan suspension. After stirring this mixture for some time the glucan particles were removed from the suspension by centrifugation. The concentration of the compound in the supernatant was determined and the amount of the compound adsorbed by the glucan particles was calculated from the difference between the starting and the end concentration of the compound.

Different classes of compounds, like proteins (e. g. 17 mg of lysozyme bound per g of glucan particles, 43 mg of myoglobin bound per g of glucan particles), flavours (e. g. 4 mg of eugenol bound per g of glucan

particles) or toxins (e. g. 2.5 mg zearalenone bound per g of glucan particles), were adsorbed by the glucan particles.

5       **Example 7:**

**Glucan particles as carrier**

10       Retinol (370 mg) was melted and glucan particles (3.0 g) were added gradually within 10 minutes under stirring at 75 °C. Stirring was continued for 10 minutes. After cooling a yellow, free flowing powder was obtained.

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## C L A I M S

1. Glucan particles with a molecular weight ( $M_w$  or  $M_n$ ) of more than 100 000 after solubilization by carboxymethylation having preserved porous and fibrous structural properties.
2. Glucan particles according to claim 1 with a size range of 0.1 to 25 micrometers, preferably 0.5 to 15 micrometers, and most preferably 2 to 10 micrometers.
3. Glucan particles according to claims 1 or 2, which are insoluble in water and in most of the known organic solvents, having activated pores and which are showing an increased stability at high pH-values.
4. Glucan particles according to claims 1 to 3 with gel forming properties.
5. Glucan particles according to at least one of the claims 1 to 4 forming a stable gel in organic solvents or acidified water, when an aqueous suspension of these particles is heated to a temperature higher than 80 °C.
6. Process for isolating glucan particles from unicellular organisms or glucan-containing plants, comprising the steps
  - a) extracting mannoproteins with water at temperatures above the boiling point of water and/or treatment of the raw materials or of the product from step a)
  - b) with proteases or by non-denaturating chemical means for the removal of proteins and/or
  - c) with lipases, or by solvent extraction for the removal of lipids

on condition that steps b) and c) may be proceeded in any sequence and if necessary in dependence on the starting material one or two steps may be left out.

- 5 7. Process according to claim 6 comprising as step a) treatment with water at temperatures above the boiling point of water under elevated pressure and adjusted pH for extracting mannoprotein.
- 10 8. Process according to at least one of the claims 6 or 7 wherein in step a) mannoproteins are extracted from an aqueous cell wall suspension in the concentration range of 1 – 20 % by weight, preferably 10 – 20 %, most preferably 13 – 17 %, with adjusted pH in the range of 5 - 9, preferably pH 6 – 8 , most preferably pH 6.5 – 7.5, at a temperature in the range of 120 – 130 °C for a defined period of time in the range of 0.1 - 24 hours, preferably 1 - 10 hours, most preferably 3 – 7 hours under elevated pressure.
- 15 9. Process according to at least one of the claims 6 - 8, wherein the mannoprotein is removed from the recovered aqueous solution (water extracts) from step a) by adding and mixing with an alcohol and precipitating.
- 20 10. Process according to claim 9 wherein the alcohol is selected from the group methanol, ethanol, propanol, and butanol.
- 25 11. Process according to claim 6 comprising as step b) treatment with proteases after pH adjustment at optimal level for the removal of contaminating proteins.
- 30 12. Process according to claim 6 wherein in step b) protein contaminants are extracted either with aqueous alkaline solution selected from the group sodium carbonate and sodium hydrogen carbonate at low concentration and low temperature  
or  
with sodium dodecylsulfate at a concentration in the range of 0.1 – 5 % by weight, preferably 1 – 3 % by weight, most preferably 1.5 - 2.5 % by weight.
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13. Process according to claim 6 comprising as step c) treatment with lipases after pH adjustment at optimal level for removal of contaminating lipids.
- 5 14. Process according to claim 6 wherein in step c) lipid contaminants are extracted either with organic solvents, that are miscible with water, selected from the group acetone ethanol, methanol, isopropanol and butanol or mixtures thereof;  
or  
10 with organic solvents that are not miscible with water, selected from the group isobutylketone, hexane, chloroform, methylenechloride, tetrachloroethylene and ethylacetate or mixtures thereof,  
or  
15 with mixtures of organic solvents that are miscible with water with organic solvents that are not miscible with water;  
or  
extraction with supercritical CO<sub>2</sub>,  
or  
extraction with supercritical CO<sub>2</sub> and organic solvents as modifiers.
- 20 15. Process according to claims 6 and 14 wherein in step c) lipid contaminants are extracted with a mixture of methanol and chloroform in a ratio of volume of 1 : 1 or of hexane and isopropanol in a ratio of volume of 3 : 2.
- 25 16. Mannoprotein obtained from step a) according to at least one of the claims 6 - 10.
17. Glucan particles obtainable by a process according to at least one of the claims 6 - 8, 11 - 15.
- 30 18. Glucan particles obtainable by a process according to claims 6 - 8 by protease treatment after extracting mannoproteins with water.
- 35 19. Glucan particles obtainable by a process according to at least one of the claims 6 - 8, 11, 13, by lipase treatment or solvent extraction after extracting mannoprotein with water and removing protein contaminants with protease.

20. Use of glucan particles according to at least one of the claims 1 - 5, 17 - 19 or obtainable from a process according to at least one of the claims 6 - 8 or 11 - 15 as adsorbents and carriers, as chromatographic active material or as adsorbents for toxic environmental compounds.  
5
21. Use of glucan particles according to at least one of the claims 1 - 5, 17 - 19 or obtainable from a process according to at least one of the claims 6 - 8 or 11 - 15 and/or mannoprotein according to claim 16 for the preparation of pharmaceutical or cosmetic formulations or as additive in food and feed or in products needed in agriculture in crop protection.  
10
22. Use of glucan particles according to at least one of the claims 1 - 5, 17 - 19 or obtainable from a process according to at least one of the claims 6 - 8 or 11 - 15 and/or mannoprotein according to claim 16 for the preparation of pharmaceutical formulations with immune system activating properties.  
15
23. Use of glucan particles according to at least one of the claims 1 - 5, 17 - 19 or obtainable from a process according to at least one of the claims 6 - 8 or 11 - 15 and/or mannoprotein according to claim 16 for the preparation of pharmaceutical formulations with antitumor activity or for administration in combination with chemotherapy or dialysis.  
20
24. Use of glucan particles according to at least one of the claims 1 - 5, 17 - 19 or obtainable from a process according to at least one of the claims 6 - 8 or 11 - 15 and/or mannoprotein according to claim 16 for the preparation of pharmaceutical formulations to improve the host-defences to bacterial or virus infections.  
25
25. Use of glucan particles according to at least one of the claims 1 - 5, 17 - 19 or obtainable from a process according to at least one of the claims 6 - 8 or 11 - 15 and/or mannoprotein according to claim 16 for the preparation of pharmaceutical formulations with prophylactic activity against diseases of age.  
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26. Use of glucan particles according to at least one of the claims 1 - 5, 17 - 19 or obtainable from a process according to at least one of the claims 6 - 8 or 11 - 15 and/or mannoprotein according to claim 16 for the preparation of formulations with cholesterol reduction activity.

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27. Use of glucan particles according to at least one of the claims 1 - 5, 17 - 19 or obtainable from a process according to at least one of the claims 6 - 8 or 11 - 15 and/or mannoprotein according to claim 16 for the preparation of formulations with an glucose regulating effect.

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28. Use of glucan particles according to at least one of the claims 1 - 5, 17 - 19 or obtainable from a process according to at least one of the claims 6 - 8 or 11 - 15 and/or mannoprotein according to claim 16 for the preparation of formulations with improving effects on cardiovascular diseases, on autoimmune conditions like HIV, arthritis, lupus, allergic asthma or multipe sclerosis.

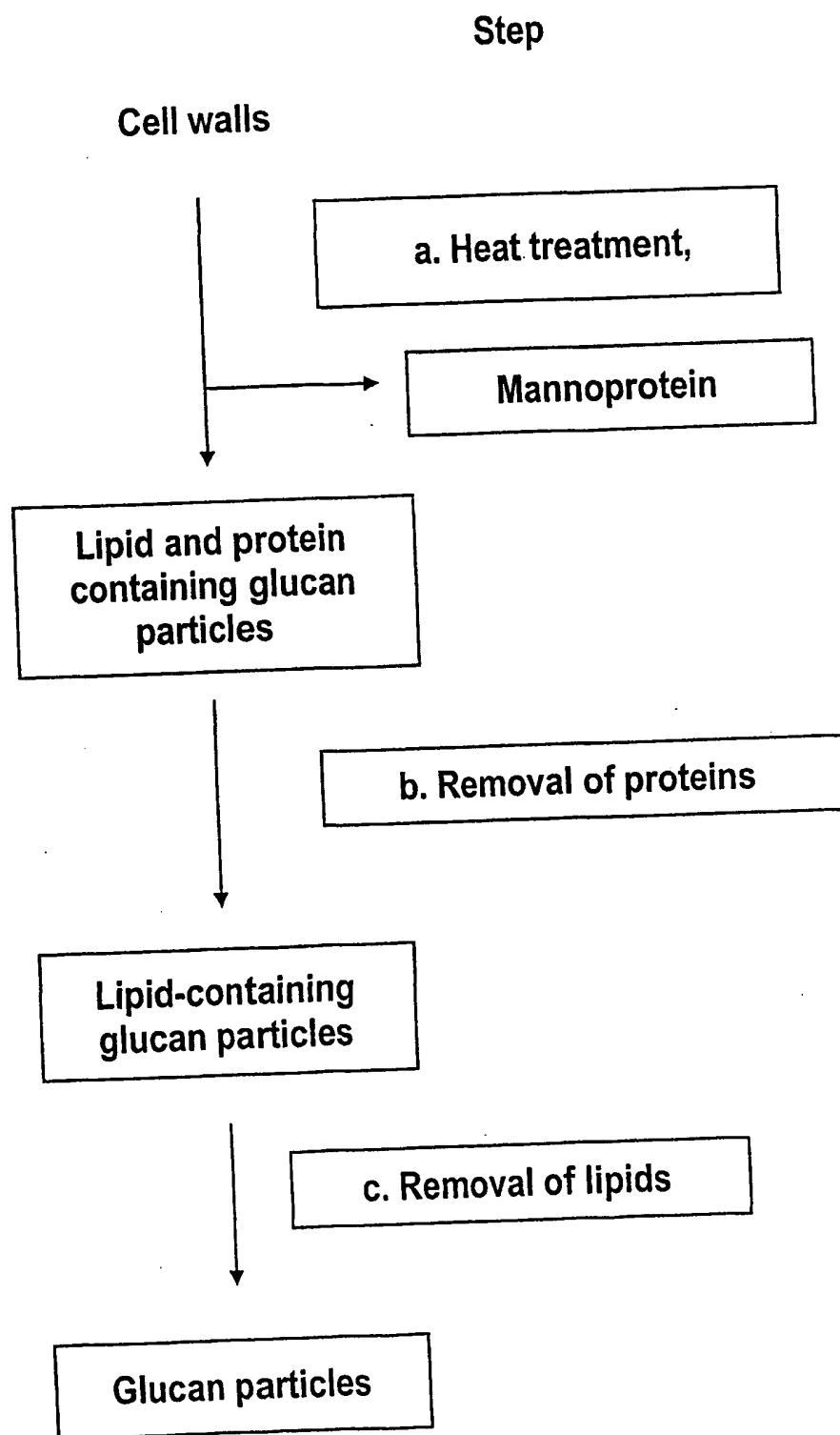
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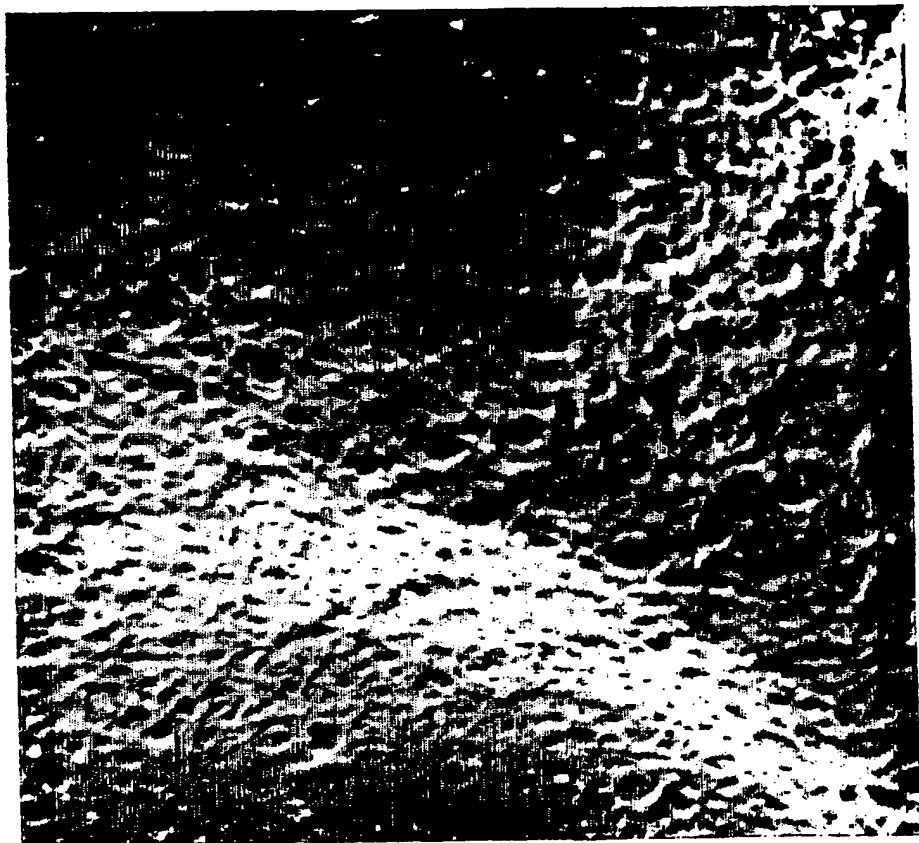
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**Fig. 1****Basic steps for glucan particle preparation from yeast**

**Fig. 2**

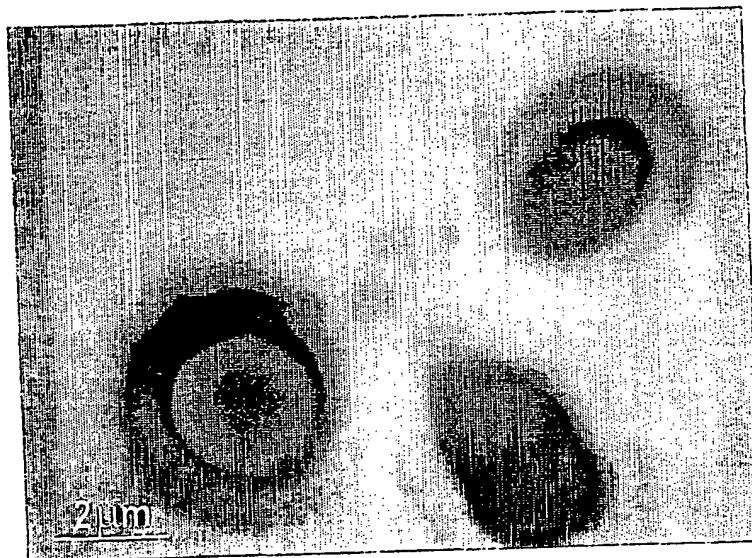
Electronmicrograph of the surface of a glucan particle according To Example 2b, acetone-treated sample; side length 1.08  $\mu\text{m}$ .



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**Fig. 3**

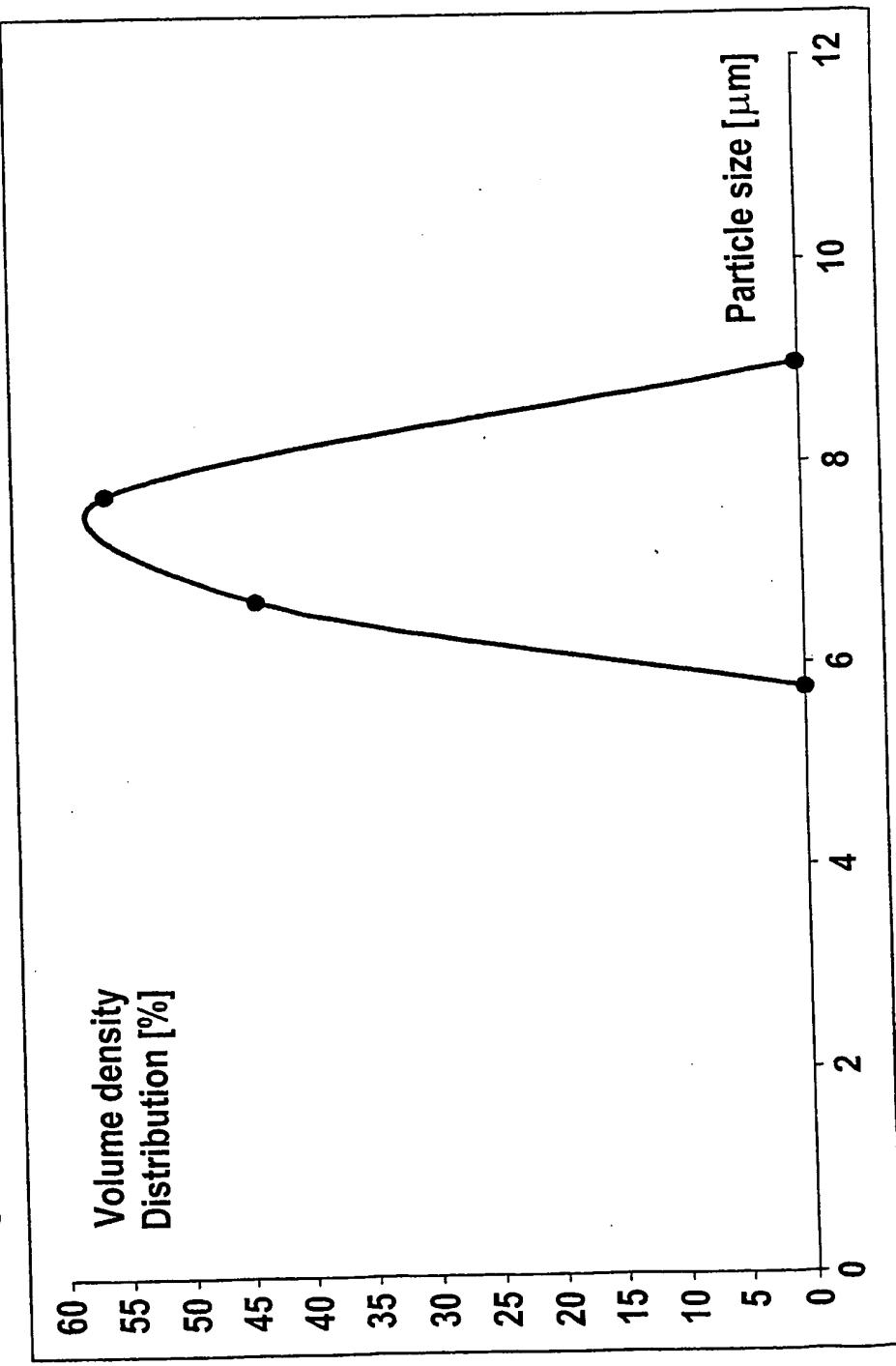
Confocal fluorescence microscopy image of lipid-containing Glucan particles according to Example 2b.

**Fig. 4**

Confocal fluorescence microscopy image after crosslinking And carboxymethylation of acetone-treated glucan particles according to Example 2b.

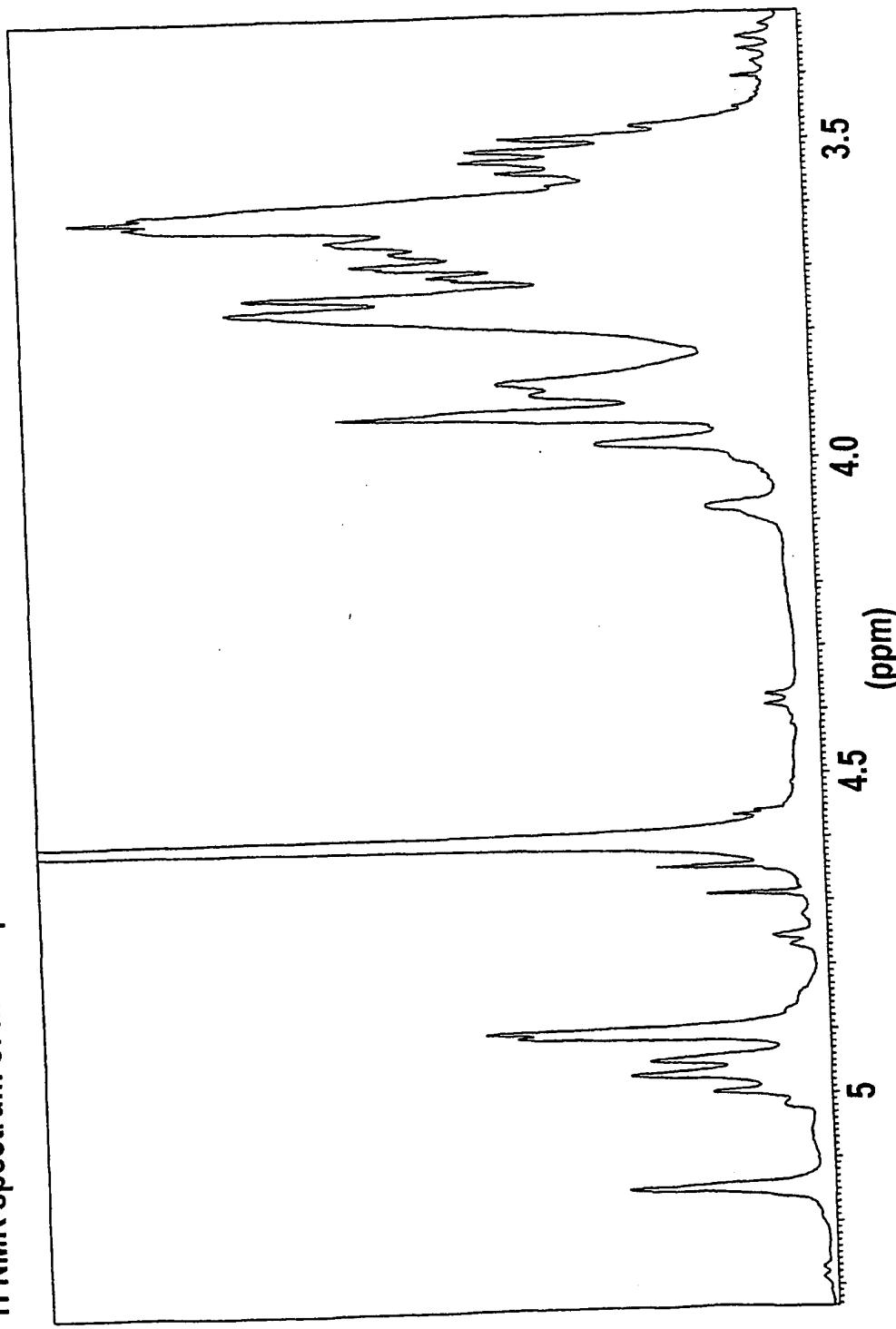


**Fig. 5**  
Particle size distribution of acetone-treated glucan particles  
according to Example 2b in water

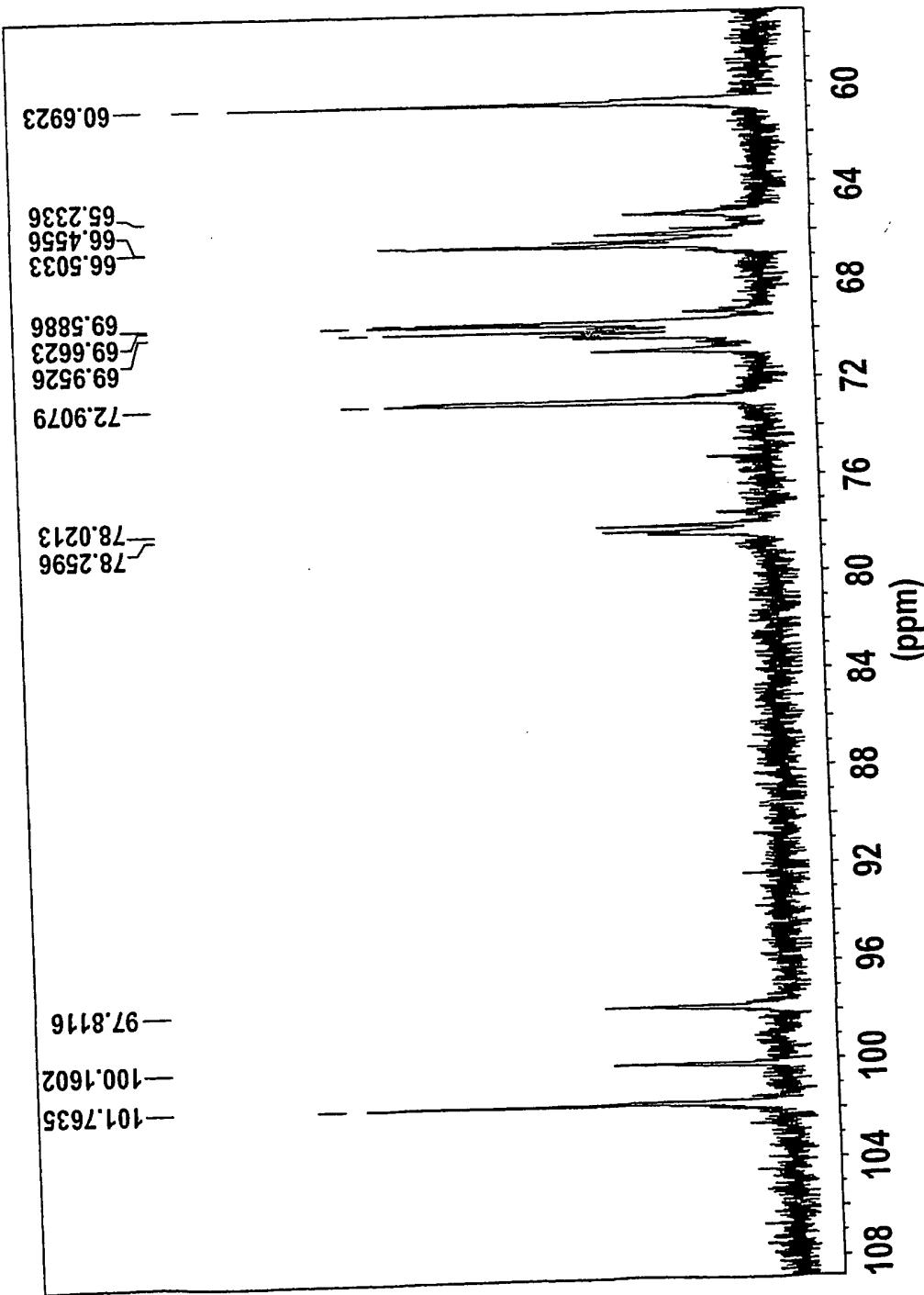


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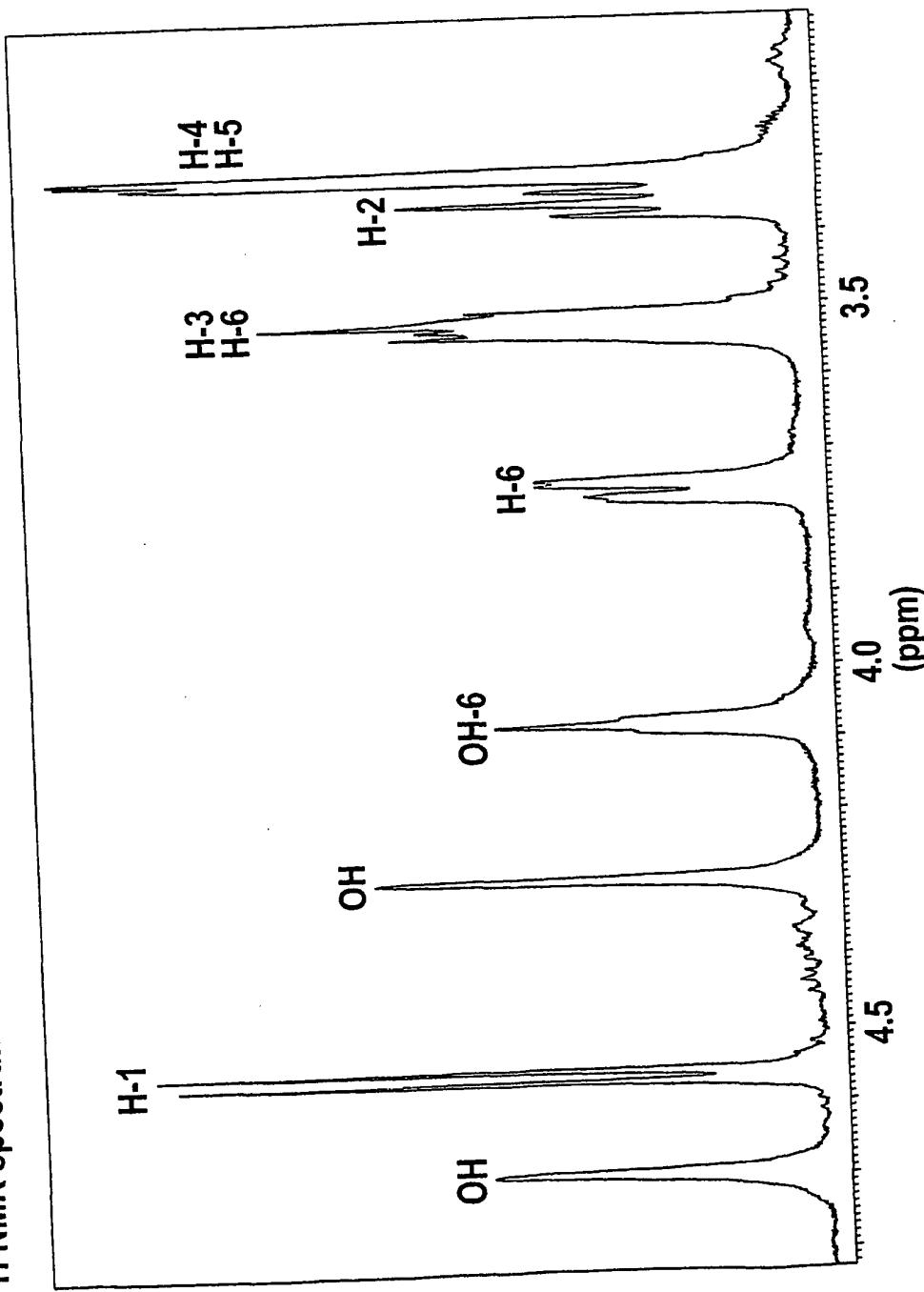
**Fig. 6**  
 $^1\text{H}$  NMR spectrum of mannoprotein according to Example 1 in  $\text{D}_2\text{O}$ .



**Fig. 7**  
 $^{13}\text{C}$  NMR spectrum of mannanprotein according to Example 1 in  $\text{D}_2\text{O}$ .

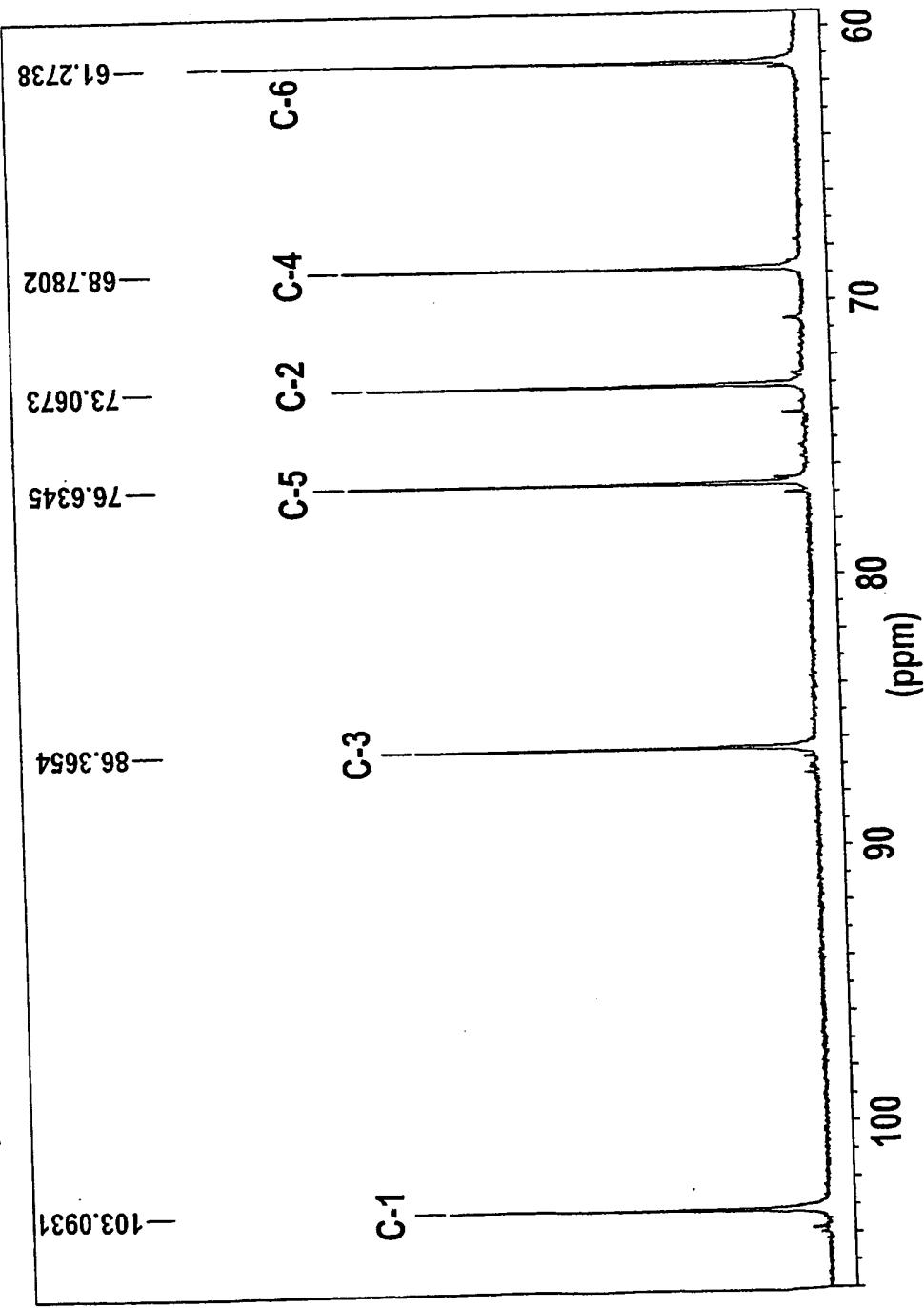


**Fig. 8**  
 $^1\text{H}$  NMR spectrum of acetone-treated glucan particles according to Example 2b in  $\text{D}_6\text{-DMSO}$ .



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**Fig. 9**  
 $^{13}\text{C}$  NMR spectrum of acetone-treated glucan particles according to Example 2b in  $\text{D}_6\text{-DMSO}$ .



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(54) Title: ISOLATION OF GLUCAN PARTICLES AND USES THEREOF

(57) Abstract: The present invention relates to the isolation of novel glucan particles but also to mannoprotein from natural sources such as yeast cell walls, novel isolation methods, and the use of products thereof.

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PCT/EP 01/08851	

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7	C08B37/00	G01N30/48	A61K31/716	A61K7/00	A23L1/03
	A23B7/00	A23K1/00			

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>L. SCARINGI ET AL.: "Cell wall components of <i>Candida albicans</i> as immunomodulators: Induction of natural killer and macrophage-mediated peritoneal cell cytotoxicity in mice by mannoprotein and glucan fractions." <i>JOURNAL OF GENERAL MICROBIOLOGY</i>, vol. 134, 1988, pages 1265-1274, XP001061263</p> <p>uk abstract page 1266, line 17 - line 36</p> <p>---</p> <p>-/-</p>	1,6-10, 14-17, 21-24

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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PCT/EP 01/08851

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 010, no. 049 (C-330), 26 February 1986 (1986-02-26) & JP 60 196195 A (DAINIHON SEITOU KK), 4 October 1985 (1985-10-04) abstract & DATABASE WPI Week 198546 Derwent Publications Ltd., London, GB; AN 1985-286695 & JP 60 196195 A (DAINIPPON SEITO KK), 4 October 1985 (1985-10-04) abstract ---	1,3,4,6, 11,17-21
X	WO 94 03500 A (CONSIGLIO NAZIONALE DELLE RICERCHE) 17 February 1994 (1994-02-17)  claims; examples ---	1,6-8, 12,17, 21-24
X	WO 91 07091 A (DONZIS B.) 30 May 1991 (1991-05-30) claims; examples 1,2 ---	1,21,25
X	PATENT ABSTRACTS OF JAPAN vol. 004, no. 026 (C-001), 6 March 1980 (1980-03-06) & JP 55 000709 A (KIRIN BREWERY CO LTD), 7 January 1980 (1980-01-07) abstract ---	1,3-5, 17,21
A	PATENT ABSTRACTS OF JAPAN vol. 011, no. 229 (C-436), 25 July 1987 (1987-07-25) & JP 62 040262 A (SANSU SEIKA KK), 21 February 1987 (1987-02-21) abstract ---	1,25
X	EP 0 416 343 A (CONSIGLIO NAZIONALE DELLE RICERCHE) 13 March 1991 (1991-03-13) the whole document ---	1-3,6-8, 17,21-25
A	EP 0 440 725 A (ALPHA-BETA TECHNOLOGY) 1 June 1994 (1994-06-01) claims ---	1,21,26
A	WO 92 07064 A (GB BIOTECHNOLOGY LIMITED) 30 April 1992 (1992-04-30) abstract page 5, line 13 - line 16 -----	1,20,21

## INTERNATIONAL SEARCH REPORT

Information on patent family members

In International Application No

PCT/EP 01/08851

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
JP 60196195	A 04-10-1985	JP 1756224 C JP 4041997 B		23-04-1993 10-07-1992
WO 9403500	A 17-02-1994	IT 1256035 B AU 4706393 A CN 1082056 A WO 9403500 A1 EP 0654047 A1 MX 9304793 A1 ZA 9305730 A		21-11-1995 03-03-1994 16-02-1994 17-02-1994 24-05-1995 31-05-1994 03-03-1994
WO 9107091	A 30-05-1991	US 5223491 A CA 2072145 A1 DE 69029860 D1 DE 69029860 T2 EP 0500718 A1 ES 2097764 T3 JP 5504131 T US 5397773 A WO 9107091 A1		29-06-1993 10-05-1991 13-03-1997 12-06-1997 02-09-1992 16-04-1997 01-07-1993 14-03-1995 30-05-1991
JP 55000709	A 07-01-1980	JP 1358381 C JP 61020561 B		13-01-1987 22-05-1986
JP 62040262	A 21-02-1987	NONE		
EP 416343	A 13-03-1991	IT 1232310 B CA 2023496 A1 EP 0416343 A2 HU 59726 A2 JP 3119995 A PL 286735 A1		28-01-1992 05-03-1991 13-03-1991 29-06-1992 22-05-1991 20-05-1991
EP 440725	A 14-08-1991	US 4962094 A DE 68915773 D1 DE 68915773 T2 EP 0440725 A1 US 6143731 A US 6020324 A AT 106193 T AU 643670 B2 AU 4492489 A CA 2001754 A1 IE 64707 B1 IL 92098 A JP 4505997 T MX 168000 B NZ 231176 A WO 9004334 A1 ZA 8908174 A		09-10-1990 07-07-1994 03-11-1994 14-08-1991 07-11-2000 01-02-2000 15-06-1994 25-11-1993 14-05-1990 28-04-1990 23-08-1995 12-04-1994 22-10-1992 27-04-1993 26-05-1997 03-05-1990 30-10-1991
WO 9207064	A 30-04-1992	AT 180829 T AU 659417 B2 AU 8724891 A BG 60684 B1 BG 97637 A CA 2094277 A1 DE 69131296 D1		15-06-1999 18-05-1995 20-05-1992 29-12-1995 29-04-1994 18-04-1992 08-07-1999

## INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No  
PCT/EP 01/08851

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9207064	A	DE	69131296 T2	30-09-1999
		DK	553176 T3	15-11-1999
		EE	3059 B1	15-02-1996
		EP	0553176 A1	04-08-1993
		ES	2132094 T3	16-08-1999
		FI	931737 A	26-05-1993
		WO	9207064 A1	30-04-1992
		GR	3031060 T3	31-12-1999
		HU	69976 A2	28-09-1995
		HU	218604 B	28-10-2000
		IE	913567 A1	22-04-1992
		JP	6504191 T	19-05-1994
		LV	10301 A , B	20-10-1994
		MX	9101622 A1	05-06-1992
		NO	931422 A	20-07-1993
		NZ	240266 A	26-03-1996
		NZ	250731 A	27-02-1996
		PT	99255 A , B	31-08-1992
		RO	113369 B1	30-06-1998
		RU	2095408 C1	10-11-1997
		US	5968811 A	19-10-1999